

Intracellular Delivery of an Antibody Targeting Gasdermin-B Reduces HER2 Breast Cancer Aggressiveness



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Abstract

Purpose: Gasdermin B (GSDMB) overexpression/amplification occurs in about 60% of HER2 breast cancers, where it promotes cell migration, resistance to anti-HER2 therapies, and poor clinical outcome. Thus, we tackle GSDMB cytoplasmic overexpression as a new therapeutic target in HER2 breast cancers.

Experimental Design: We have developed a new targeted nanomedicine based on hyaluronic acid–biocompatible nanocapsules, which allow the intracellular delivery of a specific anti-GSDMB antibody into HER2 breast cancer cells both *in vitro* and *in vivo*.

Results: Using different models of HER2 breast cancer cells, we show that anti-GSDMB antibody loaded to nanocapsules has significant and specific effects on GSDMB-overexpressing cancer cells' behavior in ways such as (i) lowering the *in vitro* cell migration induced by GSDMB;

(ii) enhancing the sensitivity to trastuzumab; (iii) reducing tumor growth by increasing apoptotic rate in orthotopic breast cancer xenografts; and (iv) diminishing lung metastasis in MDA-MB-231-HER2 cells *in vivo*. Moreover, at a mechanistic level, we have shown that AbGB increases GSDMB binding to sulfatides and consequently decreases migratory cell behavior and may upregulate the potential intrinsic procell death activity of GSDMB.

Conclusions: Our findings portray the first evidence of the effectiveness and specificity of an antibody-based nanomedicine that targets an intracellular oncoprotein. We have proved that intracellular-delivered anti-GSDMB reduces diverse pro-tumor GSDMB functions (migration, metastasis, and resistance to therapy) in an efficient and specific way, thus providing a new targeted therapeutic strategy in aggressive HER2 cancers with poor prognosis.

Introduction

Around 20% of breast tumors show *ErbB2/HER2* oncogene overexpression/amplification. This feature is usually associated with aggressiveness and poor prognosis (1, 2). HER2 tumors are treated with targeted anti-HER2 therapies (mostly antibodies like trastuzumab and pertuzumab; refs. 3, 4). However, innate or acquired drug resistance mechanisms frequently show up and

lead to disease progression (5). In addition, the acquisition of a tumor-invasive and metastatic behavior also has a negative impact on the clinical outcome for patients (6). It has been shown that coamplification of other genes in the proximity of *ERBB2/NEU* locus (17q12-21) can modulate HER2 cancer biology (7). Therefore, identification of these coamplified genes, which affects cancer development and drug response, could be a suitable

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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Clin Cancer Res 2019;25:4846–58

doi: 10.1158/1078-0432.CCR-18-2381

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Translational Relevance

To increase cancer patients' survival, it is essential to identify and validate novel cancer-specific molecular targets and try to tackle those through modern technological approaches. In this work, we have addressed these issues by validating gasdermin-B (GSDMB) cytoplasmic overexpression as a novel molecular target in HER2 breast cancers and attacking this alteration with an innovative approach: intracellular delivery of a functional anti-GSDMB antibody by nanocapsules. Our results reveal, for the first time, two important advances for translational cancer research: (i) an anti-GSDMB nanotherapy with multiple anti-tumor functions *in vitro* and *in vivo* (reducing cell migration/invasion, metastatic behavior, and drug resistance to anti-HER2), which provides a new therapeutic approach in HER2 cancer patients with poor clinical outcome, and (ii) the feasibility of targeting intracellular oncoproteins with functional therapeutic antibodies loaded on biocompatible nanocarriers, thus opening new paths for anticancer nanomedicine.

starting point for developing new therapeutic strategies for these cancer patients. In this sense, we have shown that Gasdermin B (GSDMB) overexpression/amplification is a new marker of metastasis and poor prognosis, and is also linked to reduced therapeutic response of HER2 breast carcinomas independently of their estrogen receptor status (8). GSDMB overexpression in breast cancer cells promotes aggressiveness in multiple ways such as increasing migration/invasion and supporting metastasis and resistance to anti-HER2 therapy (8, 9); thus, it is an attractive potential therapeutic target. Indeed, high levels of GSDMB expression are also associated with tumor aggressiveness in gastric, hepatic, colon, and cervical cancers (10–12). GSDMB belongs to the Gasdermin family of cytoplasmic proteins (formed by GSDMA-E and DFNB59) which can mediate either protumor or anticancer effects (13). Recent evidences indicate that they may also share the function of inducing diverse types of cell death (apoptosis, pyroptosis, or necrosis (14–18). This procell death function, characterized in detail only for GSDMD and GSDME, is activated in response to specific damaging stimuli (e.g., infections or chemotherapeutic agents) and relies on the release/exposure of their N-terminal domain, which otherwise is inhibited by their C-term domain. Cleaved or exposed N-terminal region binds to specific lipids in the cell membrane and intracellular organelle and produces lytic cell death (14–18). Although its precise physiologic acting mechanism is currently unknown, some reports indicate so far that released GSDMB N-terminal region can also produce this type of cell death (17, 19).

Because cytoplasmic GSDMB promotes multiple protumor effects and potentially has an "activatable" procell death activity, in this work, we are focusing on tackling its biological functions by a therapeutic anti-GSDMB antibody (AbGB, 8). However, up to now, the therapeutic use of mAbs is restricted to extracellular or membrane-bound proteins (20), due to their inefficient intracellular delivery by endocytosis (21). To overcome this obstacle, we have used here biocompatible hyaluronic acid (HA) nanocapsules (NC), capable of releasing small cytostatic drugs and genetic material into cancer cells (22, 23), as vehicles for intracellular delivery of an anti-GSDMB antibody in HER2/GSDMB⁺ cancer cells.

Our results demonstrate that this new anti-GSDMB nanotherapy reduces multiple protumor GSDMB functions *in vitro* and *in vivo*, in an efficient and specific way, thus providing a new targeted therapeutic approach in aggressive HER2 cancers with poor prognosis. Moreover, our findings bring forward the first evidence of the effectiveness and specificity of an antibody-based nanomedicine, which targets an intracellular oncoprotein.

Materials and Methods

Preparation and characterization of NCs and antibody-loaded NCs

A full description of the methods and the physicochemical characterization of the empty NCs and antibody-loaded NCs is provided in the Supplementary Methods. Briefly, the 130 nm HA NCs were prepared by one-step spontaneous emulsification, and the AbGB was conjugated to the NCs by controlling the charge and the hydrophobic interactions. Moreover, nanoemulsions (NE) used as control were prepared similarly without HA functionalization.

Cell culture

The HCC1954, SK-BR-3, and BT474 human HER2 breast carcinoma cell lines were obtained from the American Type Culture Collection (ATCC), and MDA-MB-231-HER2 (24) were kindly provided by Dr. Giulio Francia (University of Texas, El Paso, Texas). The cells were authenticated by short tandem repeat profiling according to the ATCC guidelines and were routinely tested for Mycoplasma infection. Cells were cultured in RPMI-1640 or DMEM medium (Gibco), supplemented with 10% FBS (Gibco), 10 mmol/L glutamine (Life Technologies), and 1% penicillin/streptomycin (Invitrogen). Migration, cell viability proliferation assays and confocal microscope imaging studies are detailed in the Supplementary Methods. The GSDMB-silenced (sh1/sh2) and nontargeting control HCC1954 cells (shC) were described previously (9). SK-BR-3, BT474, and MDA-MB-231-HER2 cells overexpressing myc-tagged GSDMB and control empty vector, as well as derivate cells stably expressing mCherry-luciferase, used for the *in vivo* assays, were obtained by lentiviral infection, as described in the Supplementary Methods.

Animal *in vivo* studies

All the experimental procedures with mice were approved by the internal ethical research and animal welfare committee (IIB, UAM), and by the Local Authorities (Comunidad de Madrid, PROEX424/15). They complied with the European Union (Directive 2010/63/UE) and Spanish Government guidelines (Real Decreto 53/20133). The detailed description of the different *in vivo* experiments is provided in the Supplementary Methods. Briefly, orthotopic breast tumor xenografts and experimental lung metastasis assays were performed in female nu/nu mice (Charles River) following standard procedures (9, 25). For each assay type (nanotherapy tissue distribution as well as therapeutic efficacy in tumor growth and metastasis), tumor-bearing mice were administered with the different experimental treatments as specified in the Supplementary Methods. Luciferase bioluminescence emission was used for monitoring tumor progression, and tumors and other tissues at the end of the experiments were collected and analyzed as described in the Supplementary Methods.

Statistical analysis

The differences between two experimental conditions were evaluated with the Student *t* test (unpaired, two-tailed) using GraphPad 5.0. *P* value < 0.05 was considered as statistically significant. The data are presented as the mean ± SEM.

Results

Biocompatible nanocarriers' characterization and their loading with anti-GSDMB antibody

GSDMB overexpression promotes an aggressive behavior (enhanced migration, invasion, and resistance to anti-HER2 therapy) and poor clinical outcome in HER2 breast carcinomas (8, 9). In order to attack these GSDMB protumor effects, we have designed a targeted anti-GSDMB nanotherapy based on the use of a specific monoclonal anti-GSDMB antibody (AbGB; ref. 8). Bearing in mind that GSDMB is a cytoplasmic protein and mAbs cannot cross the cell membrane on their own (21), we have developed NCs in order to deliver the AbGB into cells. These NCs have been obtained by a spontaneous emulsification method that does not require the use of organic solvents, heat, or a high-energy input (26). Moreover, they are also functionalized in their surface with amphiphilic dodecylamide HA (Fig. 1A). The modification of HA with a hydrophobic chain favors its interaction with the oily core, generating a strong hydrophobic layer that is surrounded by the hydrophilic chains of HA and with a PEGylated surface (Fig. 1A). The physicochemical description revealed that NCs had a diameter of about 130 nm, a polydispersion index ≤ 0.2, a negative zeta potential of −20 mV, and were stable in human plasma (Supplementary Fig. S1); these features do ensure their biocompatibility. In addition, as controls we used NEs, which have a similar composition to NCs but without any HA functionalization (Supplementary Fig. S1C).

To test if the purified AbGB (Supplementary Fig. S2A) could be efficiently and stably loaded into NCs, we have performed two alternative methods with antibody differences in charge and hydrophobicity: protonated (pH 4.5) and neutral (pH 7.4; Supplementary Fig. S2B). Comprehensive studies showed that the association of AbGB, either protonated or neutral, to the NCs did not change the nanocarrier physicochemical properties. Moreover, a high association efficiency (>84%) was observed for both systems (Supplementary Fig. S2C and S2D). In fact, only a small change in the NCs zeta potential was observed, −10 mV for the neutral antibody and −2 mV for the protonated one (Supplementary Fig. S2E). Hence, NCs can be efficiently loaded with AbGB through mainly hydrophobic interactions, without chemical reactions or other modifications, and using a simple and scalable formulation strategy. Therefore, we have chosen a standard protocol to associate 25 μg of the neutral AbGB with 1.0 mg of NCs for further functional analysis (Supplementary Fig. S2F). Importantly, AbGB-NCs were also as stable as empty NCs in human serum after 24-hour incubation (Supplementary Fig. S2G), showing AbGB-NCs' suitability for systemic delivery *in vivo*.

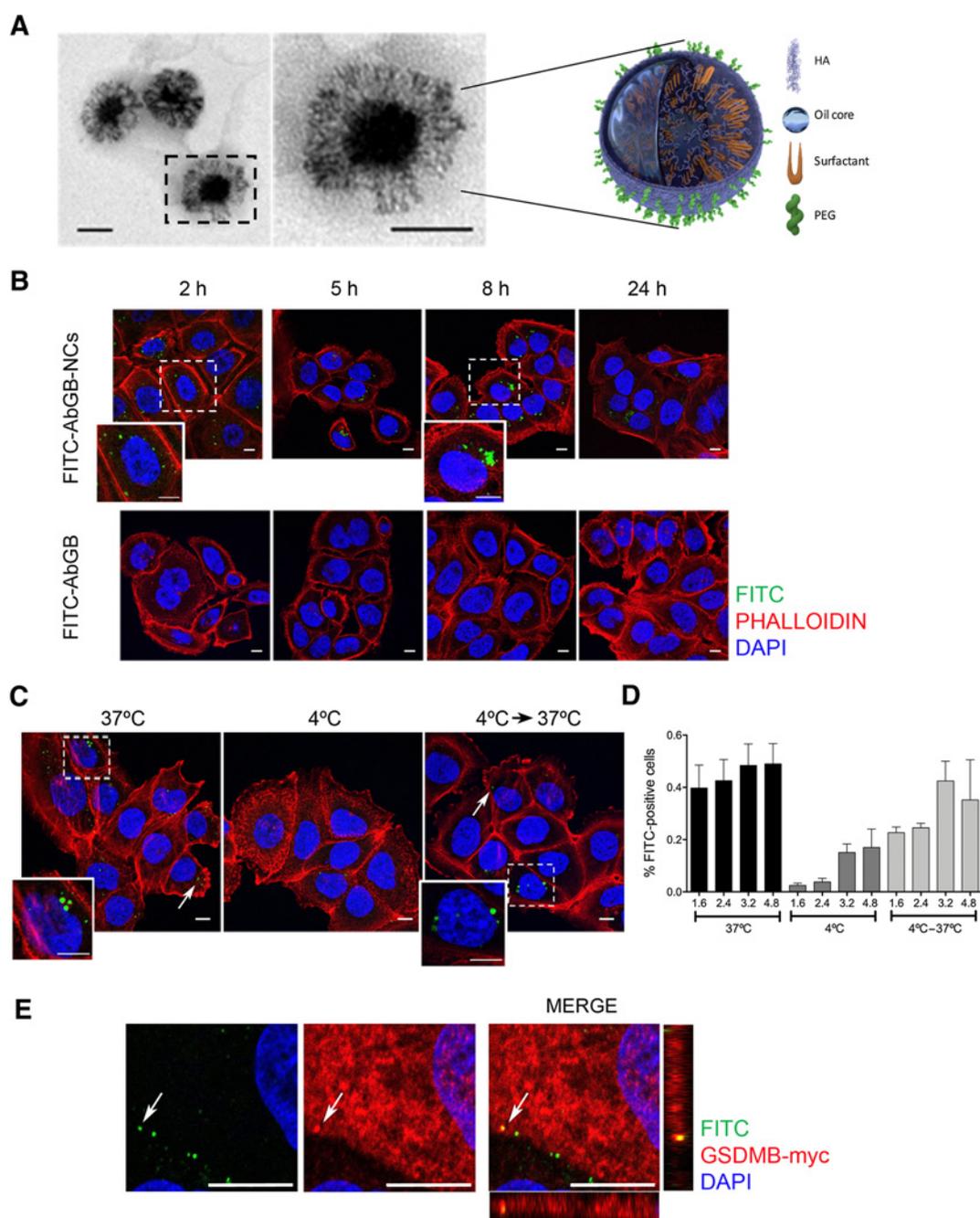
Breast carcinoma cells efficiently internalize the anti-GSDMB antibody associated with NCs

To test the NCs' and NEs' potential therapeutic utility, first we evaluated their cytotoxicity and intracellular internalization in three HER2 breast carcinoma cell lines, one with high endogenous GSDMB expression (HCC1954), and other two GSDMB-negative (SK-BR-3 and BT474; Supplementary Fig. S3A). In all of these cell

models, NCs generally showed lower cytotoxicity than NEs after 72-hour treatment; having high concentrations of NCs (0.8–1.0 mg/mL) only a small effect on viability (Supplementary Fig. S3B). Afterward, to assess the capacity of NCs to deliver intracellularly the AbGB, cell lines were treated with 0.8 to 1.0 mg/mL NCs loaded with FITC-labeled AbGB at different time points. With a single dose of FITC-AbGB-NCs, clear FITC-AbGB internalization was observed at 2, 4, and 8 hours in HCC1954, SK-BR-3, and BT474, respectively (Supplementary Fig. S3C). Orthogonal confocal images of actin-stained cells confirmed that the AbGB was indeed inside cells and not attached to the cell membrane (Supplementary Fig. S3C). Next, for each cell model, we thus optimized the protocol to allow the highest cumulative dosage after consecutive treatments for 72 hours, while keeping low toxicity (described in Supplementary Fig. S3D). Therefore, HCC1954, SK-BR-3, and BT474 cells were incubated daily with the highest tolerated dose (3.2, 0.8, and 1.0 mg/mL of NCs) for 2, 4, and 8 hours, respectively, after which the treatment medium was removed (Supplementary Fig. S3D). Then, using its optimized dosage, we thoroughly analyzed in HCC1954 cells the internalization capacity and dynamics of AbGB-NCs (Supplementary Fig. S3D) in two complementary time-course experiments. In these, we compared the AbGB-NCs and free AbGB uptake, either with FITC-labeled (experiment 1; Fig. 1B; Supplementary Fig. S4A) or -unlabeled AbGB (experiment 2; Supplementary Fig. S4A and S4B). In the latter, we detected the distribution of the AbGB by indirect immunofluorescence using an anti-mouse secondary antibody. In both tests, the maximum AbGB-NCs uptake took place at 5 to 8 hours, and AbGB was detected up to 24 hours, whereas no intracellular fluorescence was evident when cells were exposed to free AbGB (Fig. 1B; Supplementary Fig. S4A and S4B). Therefore, these results prove that AbGB association with NCs is required for its cellular uptake and demonstrate the utility of NCs for intracellular antibody delivery.

AbGB is internalized mainly by endocytosis, thereby reaching its target GSDMB protein in the cytoplasm

To check if AbGB-NCs were internalized by endocytosis, as other NCs do (27), HCC1954 cells exposed to increasing concentrations of FITC-AbGB-NCs were maintained at 4°C or 37°C (Fig. 1C and D; Supplementary Fig. S3C), according to established protocols (28). Confocal microscopy revealed a significant reduction in intracellular fluorescence in those cells grown at 4°C rather than at 37°C, in a NCs-AbGB dose-dependent concentration (Fig. 1D). Moreover, shifting the temperature from 4°C to 37°C clearly increased FITC intracellular signal (Fig. 1D; Supplementary Fig. S4C), supporting that AbGB-NCs are actively internalized by endocytosis. In order to identify the endocytic route, we assessed the colocalization of FITC-AbGB with CD44, caveolin, and clathrin proteins, which are functionally involved in endocytosis (29–31). FITC-AbGB-NCs were compared with FITC-AbGB-NEs (which lack HA, the ligand of CD44 receptor; ref. 31) to evaluate the importance of CD44 in NCs' uptake. Both NCs and NEs displayed similar overall rates of FITC-AbGB internalization (Supplementary Fig. S5D) and, surprisingly, equal degree of colocalization between AbGB and CD44-coated vesicles (Supplementary Fig. S5A and S5E). Nonetheless, colocalization of FITC-AbGB-NEs with clathrin and caveolin increased in comparison with FITC-AbGB-NCs (Supplementary Fig. S5B, S5C and S5E). These findings suggest that the HA interaction in the nanocarrier with cellular CD44 is not required for AbGB uptake,

**Figure 1.**

The HA NCs loaded with the anti-GSDMB antibody (AbGB) can be internalized by endocytosis until reaching its target protein GSDMB in breast cancer cells. **A**, A representative image of empty NCs obtained by transmission electron microscopy (left) and its magnification (middle). Scale bar, 100 nm. Right: scheme of the structural composition of the NCs, highlighting the oily core (composed of a medium chain triglyceride—Miglyol812—and a surfactant layer of polysorbate 80) and the polymeric shell (composed of polyethylene oxide surfactants, hydrophobically modified HA, and a surface of PEG-15 hydroxystearate—SolutolHS15). **B**, Confocal microscopy images showing the intracellular localization of FITC-AbGB (green) in HCC1954 cells treated for 2 hours with 3.2 mg/mL FITC-AbGB-loaded NCs (FITC-AbGB-NCs, top plots) or free FITC-AbGB (AbGB, bottom plots) and analyzed at different time points. The nuclei were stained with DAPI and the actin cytoskeleton with Alexa 647 phalloidin (red). **C** and **D**, Efficiency of FITC-AbGB-NC internalization at different temperatures (an indirect measure of endocytic uptake). Confocal microscopy images (**C**) and quantification (**D**) of the intracellular FITC-AbGB-NCs uptake in HCC1954 cells cultured under the following conditions: 2 hours at 37°C, 2 hours at 4°C, or 2 hours at 4°C followed by 2 additional hours at 37°C (to activate endocytosis). Insets, magnified images of the boxed areas (scale bar, 10 μ m). **D**, The percentage of cells with clear intracellular FITC-specific fluorescence was measured by confocal imaging. The data represent the mean \pm SEM of at least 50 cells per condition. **E**, Partial colocalization of FITC-AbGB-NCs (arrows) with the cytoplasmic GSDMB protein (red). HCC1954 cells stably expressing myc-tagged GSDMB were treated for 2 hours with FITC-AbGB-NCs. Ectopic GSDMB-myc protein was detected using an anti-myc antibody (red). Scale bar, 10 μ m.

and that different endocytic mechanisms might act depending on nanocarriers' composition and functionalization. Further analysis of vesicle trafficking (colocalization studies with intracellular markers of the endosome, lysosome, Golgi apparatus, and endoplasmic reticulum) and final intracellular destiny of FITC-AbGB-NCs (Supplementary Fig. S6) showed that about 20% of FITC-AbGB-NCs might be degraded by the lysosomal pathway (Supplementary Fig. S6A and S6B). The rest of FITC-AbGB possibly leaves the lysosomes and could enter subcellular trafficking, maturation pathways or might be released in the cytosol (Supplementary Fig. S6A and S6B). Interestingly, we observed that discrete foci of FITC-AbGB colocalize with myc-tagged cytoplasmic GSDMB in HCC1954 (Fig. 1E) and SK-BR-3 cells (Supplementary Fig. S6C), pointing out that part of FITC-AbGB finally reaches its cytoplasmic target protein.

AbGB-NCs' treatment reduces the migratory capacity and enhances the sensitivity to trastuzumab of GSDMB-positive breast cancer cells

In order to assess if AbGB-NCs could have a functional effect on GSDMB-positive cancer cells behavior, we first focused on cell migration, because we previously reported that GSDMB overexpression enhances the motility and invasiveness of MCF7 cells, whereas its shRNA silencing diminishes the migratory capacity of HCC1954 cells (9). Using wound-healing assays in HCC1954 cells, we observed that AbGB-NCs' treatment reduces (almost 20%) cell migration, whereas no effect was detected when cells were treated with either free AbGB, empty NCs, or NCs loaded with an irrelevant antibody (IgG-NCs, Fig. 2A; Supplementary Fig. S7A). Similarly, transwell migration assays in SK-BR3 and BT474 stably overexpressing GSDMB (Supplementary Fig. S3A) revealed again that AbGB-NCs but not IgG-NCs treatment significantly decreases the migration behavior specifically in GSDMB-expressing cells (Fig. 2B and C; Supplementary Fig. S7B and S7C). Interestingly, migration blockage after treatment with AbGB-NCs was not due to proliferation changes in any analyzed cell line (Supplementary Fig. S7D).

Moreover, to test the therapeutic use of AbGB-NCs, we evaluated the sensitivity of cancer cells together with trastuzumab, because GSDMB overexpression reduces the response to this anti-HER2 agent (8). For this, we treated three breast carcinoma cell models with AbGB-NCs, empty NCs, and free AbGB, in the presence/absence of trastuzumab (Fig. 2D). The combination of AbGB-NCs and trastuzumab treatment produced a significant additive effect on cell viability (20%–30% decrease) compared with trastuzumab alone, in GSDMB-expressing cells (HCC1954 shC, SK-BR-3 GB, and BT474 GB), whereas no such effect was observed in GSDMB-nonexpressing cells (HCC1954 sh1, SK-BR-3 C, and BT474 C; Fig. 2D).

Altogether, these *in vitro* data prove that antibody-loaded NCs hinder the protumor activities mediated by GSDMB and, from a therapeutic point of view, show that this anti-GSDMB nanotherapy could be used to reduce the trastuzumab resistance of GSDMB⁺ cancer cells.

Systemically administered AbGB-NCs can reach breast cancer cells *in vivo*

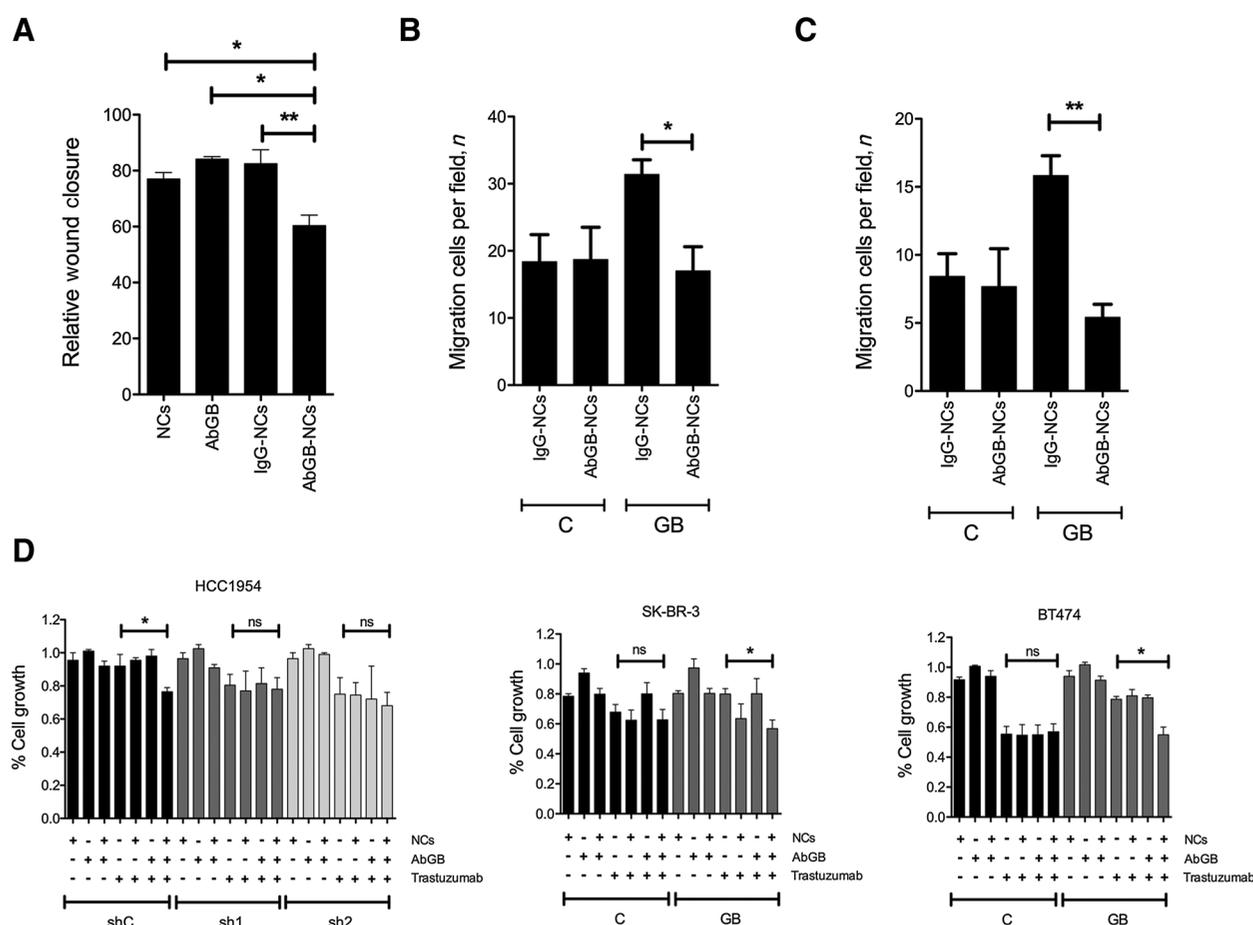
After showing that AbGB-NCs have functional effects on GSDMB⁺ cancer cells *in vitro* (Fig. 2A–C), we assessed their capacity to target breast tumor tissue *in vivo*. We evaluated tissue biodistribution of FITC-AbGB-NCs or FITC-AbGB-NEs, used as

control, in mice bearing HCC1954 breast cancer xenografts (Fig. 3A). The analysis of different tissues *ex vivo* 8 and 24 hours after treatment showed that FITC-AbGB-NCs fluorescent signal accumulated mostly in the breast tumor tissue (Fig. 3A and B), and very little in other tissues, except the liver (Fig. 3A). By contrast, FITC-AbGB loaded into NEs showed less accumulation into the breast tumor and a much wider tissue distribution (Fig. 3A and B). In fact, FITC-AbGB-NEs' accumulation in spleen and kidneys (Fig. 3A) suggests that NEs were quickly detoxified, thus limiting their *in vivo* effectiveness. Overall, these findings prove the FITC-AbGB-NCs' ability to reach effectively their target tumor tissue *in vivo*, and also their low accumulation in vital organs (heart and lung) indicates that the treatment is unlikely to produce significant systemic toxicity.

Targeted anti-GSDMB nanotherapy efficiently reduces HER2 breast tumor growth *in vivo* by enhancing cell death

Based on positive results from the biodistribution assays, we studied if AbGB-NCs could have a functional effect on tumor progression *in vivo*. It should be noted that GSDMB silencing by shRNA alone did not significantly affect cancer growth or tumor histology of HCC1954 cells (Supplementary Fig. S8A and S8B). Therefore, we hypothesized that direct interference of the GSDMB protein by the AbGB may have a stronger biological effect than the reduction in mRNA GSDMB levels. To test this possibility, we performed two complementary experiments on mice bearing HCC1954 breast cancer xenografts. In our first approach, mice left and right mammary glands were respectively inoculated with control (shC) and GSDMB-silenced cells (sh1; Supplementary Fig. S8C–S8F). Then, mice were treated with AbGB-NCs, free AbGB, and empty NCs (treatment scheme in Supplementary Fig. S8C). Remarkably, HCC1954 shC tumors treated with AbGB-NCs displayed a significant reduction in tumor size (Supplementary Fig. S8D and S8E) and luciferase bioluminescence intensity (Supplementary Fig. S8D and S8F) compared with control treatments (empty NCs or free AbGB alone). By contrast, no such differences were observed in tumors derived from sh1 cells (Supplementary Fig. S8C–S8F). Thus, these data prove that AbGB-NCs decrease tumor growth *in vivo* specifically in cancer cells with high GSDMB expression, whereas they do not affect cancer cells lacking the target protein. Next, to ensure that this *in vivo* effect was specific to AbGB antibody, in the second approach, we compared AbGB-NCs with IgG-NCs on two different HER2 cell models HCC1954 (estrogen receptor negative; Fig. 4) and BT474 (estrogen receptor positive; Supplementary Fig. S9) using a similar treatment setup to the one described above (Supplementary Fig. S8C). Again, only the AbGB-NCs but not the IgG-NCs' treatment reduced significantly the bioluminescence (Fig. 4A and B; Supplementary Fig. S9A and S9B), tumor volume, and weight (Fig. 4C and D; Supplementary Fig. S9C and S9D) exclusively of GSDMB-expressing cancer cells such as HCC1954 shC and BT474 GB.

In addition, to identify the biological mechanism underlying the delayed tumor growth produced by AbGB-NCs, we assessed on tumor sections the proliferation and apoptotic rate using proliferating cell nuclear antigen (PCNA) and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining, respectively (Fig. 4E and F; Supplementary Fig. S9E and S9F). There was a significantly higher rate of apoptosis in the GSDMB-expressing tumors (HCC1954 shC and BT474 GB) treated with AbGB-NCs compared with those tumors treated with

**Figure 2.**

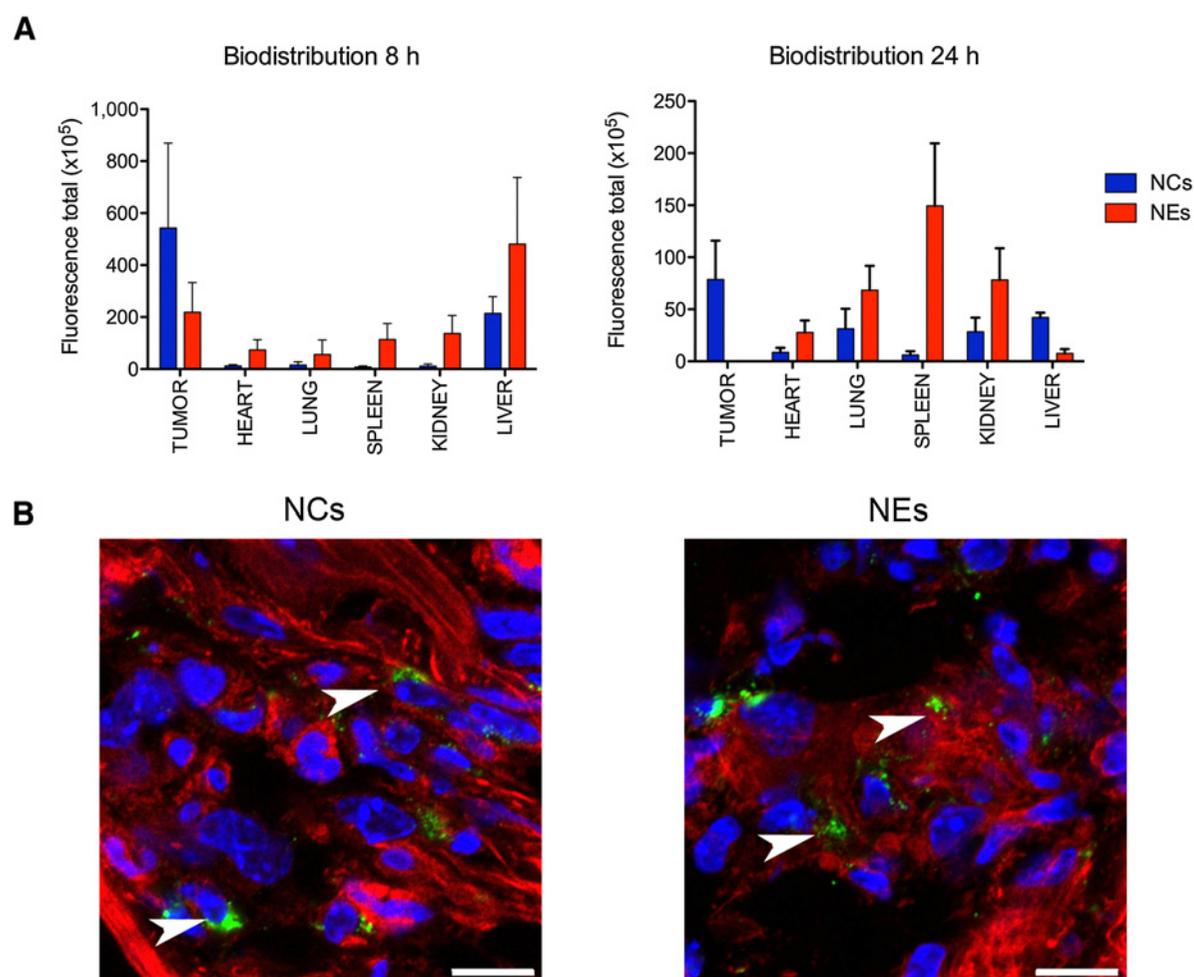
AbGB-NCs decrease cell migration and increase sensitivity to trastuzumab of HER2 GSDMB-positive cancer cells. **A**, Wound-healing assays of HCC1954 cells treated daily for 2 hours with 3.2 mg/mL of anti-GSDMB-loaded NCs (AbGB-NCs), NCs loaded with an irrelevant IgG antibody (IgG-NCs), empty NCs, or 80 μ g of anti-GSDMB alone. Quantification of the wound area covered by migrating cells at 72 hours relative to 0 hour. The data represent the mean \pm SEM of three independent experiments. **B** and **C**, Quantification of transwell migration assays of SK-BR-3 (**B**) and BT474 (**C**) cells exogenously overexpressing GSDMB (GB) or an empty control vector (C) treated daily with 0.8 mg/mL (**B**) or 1 mg/mL (**C**) of IgG-NCs or AbGB-NCs for 48 hours. **D**, Effect of AbGB-NC on the viability of cancer cells in combination with trastuzumab. Cells with high levels of GSDMB (HCC1954 shC, SK-BR-3 GB, BT474 GB) or low/negative GSDMB expression (HCC1954 sh1/sh2, SK-BR-3 C, BT474 C) were treated for 72 hours with AbGB-NCs, empty NCs, or AbGB alone, in the presence or absence of trastuzumab (200 mg/mL for HCC1954 and BT474 and 100 mg/mL for SK-BR-3, respectively). The data represent the mean \pm SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; and ns, nonsignificant.

IgG-NCs (Fig. 4F; Supplementary Fig. S9F). Furthermore, the proliferation rate was similar in all cases independently of the treatment, which points out that tumor growth reduction was due to enhanced apoptosis induction (Fig. 4F; Supplementary Fig. S9F). Again, no such effects on tumor growth, proliferation, or apoptosis induction were observed in GSDMB-low tumors (HCC1954 sh1 or BT474 C) in any given treatment condition, corroborating AbGB-NCs' nanotherapy *in vivo* specificity (Fig. 4; Supplementary Fig. S9). Moreover, Western blot analyses of the tumors prove that AbGB did not affect total GSDMB protein levels (Fig. 4G), suggesting that the antibody modulates protein function rather than inducing its degradation.

AbGB-NC treatment decreases breast cancer metastasis *in vivo*

The strong therapeutic effect of AbGB-NCs on tumor growth led us to analyze if this treatment could also block metastatic spread, given the reported association of GSDMB overexpression with

metastatic potential in MCF7 cells (9) and HER2 breast cancer biopsies (8). For this reason, we performed experimental lung metastasis assays using MDA-MB-231-HER2 (24) cells with or without stable GSDMB expression (Supplementary Fig. S10A). Prior to these experiments, we identified on these cells the conditions for low NCs cytotoxicity (Supplementary Fig. S10B) and optimized AbGB-NCs internalization (Supplementary Fig. S10C). A week after cell inoculation by tail vein injection, mice were treated with either AbGB-NCs or IgG-NCs for 3 additional weeks. Consistently with the prometastatic effect of GSDMB in other models (9), animals inoculated with GSDMB-overexpressing cells (GB) showed stronger bioluminescence signal and bigger lung metastatic foci in comparison with MDA-MB-231-HER2 control cells (C; Fig. 5). Interestingly, AbGB-NCs' treatment reduced the bioluminescence signal (Fig. 5A and B) and metastatic foci size (Fig. 5C) in GSDMB-overexpressing cells, whereas no such effects were detected in control cells (C).

**Figure 3.**

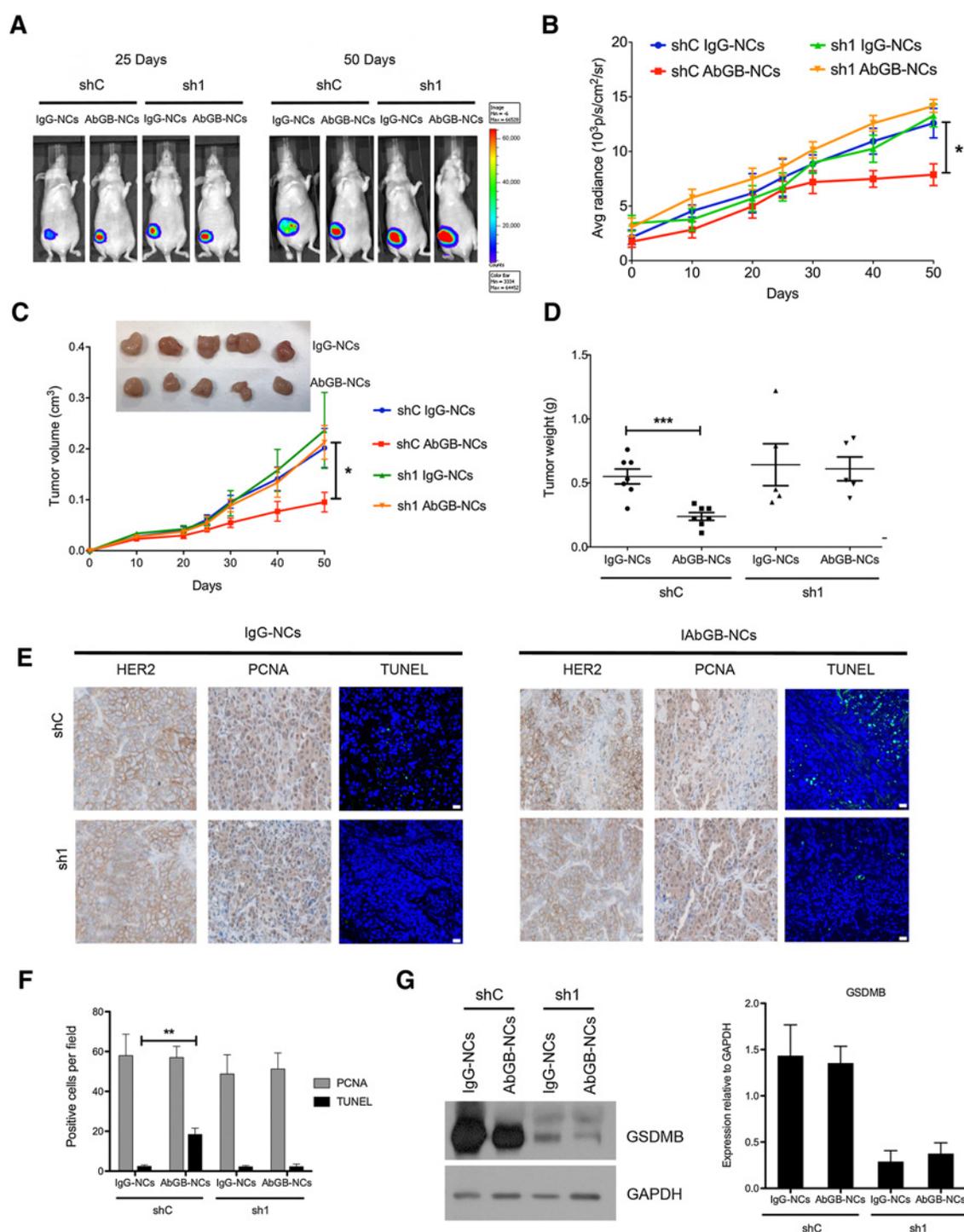
AbGB-NCs efficiently target breast tumors *in vivo*. **A** and **B**, Biodistribution of FITC-AbGB-NCs and FITC-AbGB-NEs (nanoemulsions, lacking HA) in mice bearing orthotopic xenografted HCC1954 breast tumors. Animals were treated with FITC-AbGB-NCs or NEs (4.17 mg/kg of AbGB loaded on 200 mg/kg NCs/NEs) when the breast tumors reached 0.7 cm. **A**, Quantification of the FITC fluorescence (normalized to the background) of the indicated organs *ex vivo* after 8- and 24-hour treatment with FITC-AbGB-NCs or FITC-AbGB-NEs. The bars represent the mean fluorescence intensity \pm SEM from 3 mice per condition. **B**, Representative confocal microscope images of breast tumor cryosections stained with phalloidin (F-actin; red) and DAPI (blue), demonstrating the intracellular uptake (arrows) of FITC-AbGB-NCs and FITC-AbGB-NEs *in vivo* (green). Scale bar, 10 μ m.

Taken together, all the *in vivo* data demonstrate that the intracellularly delivered AbGB reduces tumor growth and metastatic ability of HER2⁺/GSDMB⁺ breast cancer cells (independent of hormonal receptor status), thus validating its new therapeutic potential in these aggressive tumors.

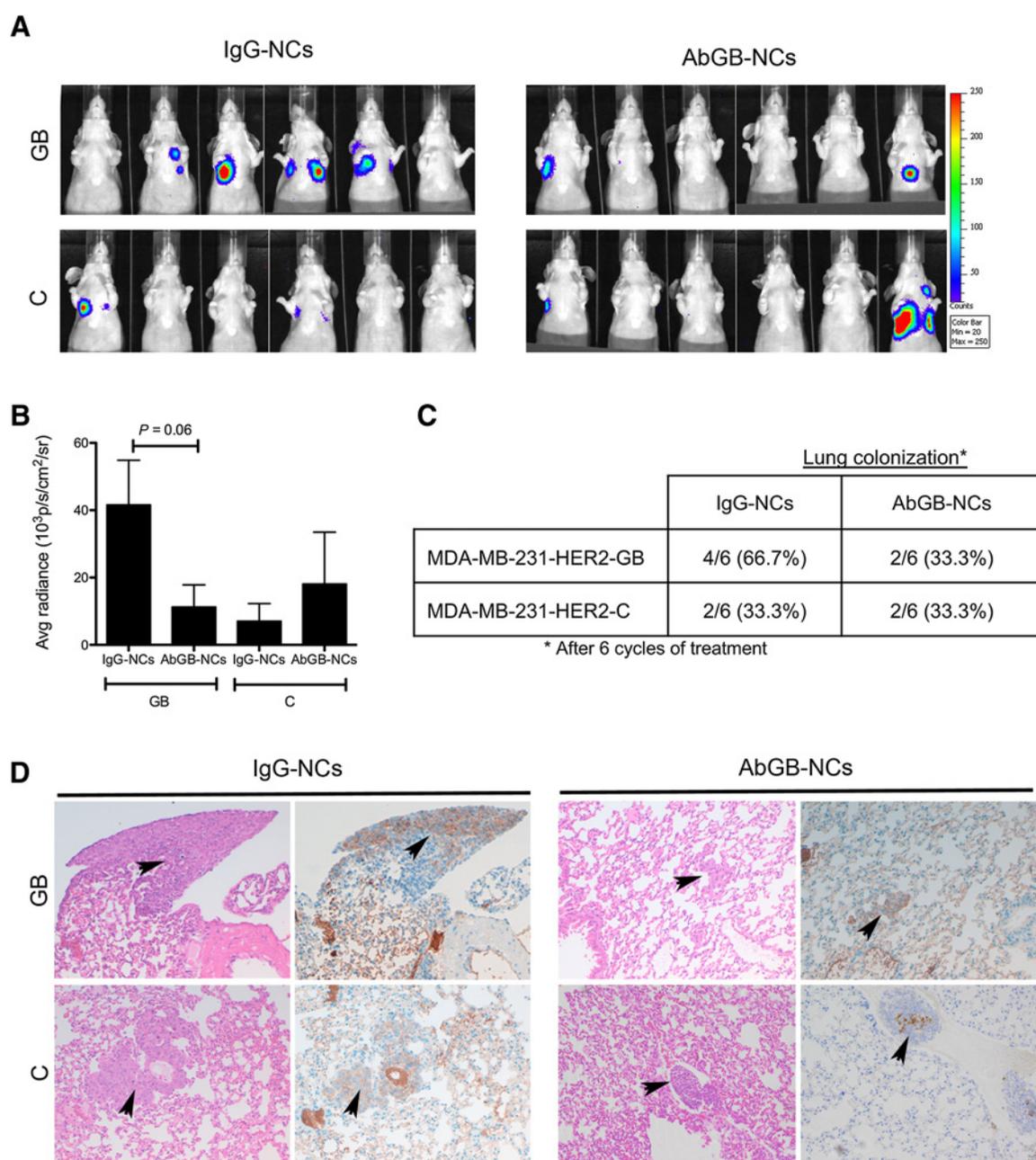
AbGB-NC treatment reinforces GSDMB binding to sulfatide

Finally, we investigated the mechanism by which AbGB affects GSDMB protein function and consequently regulates the GSDMB-dependent biological processes. Other gasdermin proteins have been reported to exist in two alternative states; in the closed/autoinhibited state, the C-terminal and N-terminal domains adopt a conformation that prevents the binding of phospholipid ligands (13, 14). Following specific stimuli or changes in cellular conditions, each of these proteins adopts an open conformation or undergoes cleavage that exposes the N-terminal domain, which is involved in the binding to cell membrane lipids causing ultimately cell death (13, 14, 32).

By analogy, we hypothesized that interaction of AbGB to GSDMB enhances lipid binding by altering the protein conformation. In fact, the *in silico* modeling of the 3D interaction of GSDMB protein with AbGB revealed that antibody binds to a region comprising part of the flexible interdomain linker and the ensuing C-terminal domain amino acid residues, suggesting that it is likely to affect the autoinhibitory interface between N-term and C-term domains (Supplementary Fig. S10D) and thus alter GSDMB binding to lipids. To test this possibility, we focused on the reported interaction of GSDMB with sulfatides (33), as these lipids are involved in multiple key cancer processes such as some cell death mechanisms, cell migration, or metastasis (34). Using protein lipid overlay assays, we observed that preincubation of the purified GSDMB protein (33) with AbGB increased the GSDMB binding to sulfatide, especially when a low concentration of these lipids is given (Fig. 6A and B). This suggests that the binding of AbGB brings along a GSDMB conformation change and eases the

**Figure 4.**

AbGB-NCs reduce tumor growth *in vivo* by increasing cell death rate specifically in GSDMB-positive HCC1954 breast tumors. **A**, Representative images of tumor size (measured by luciferase bioluminescence) at the beginning of the treatment (day 25) and at the end of the experiment (day 50). Mice were inoculated with either HCC1954-mCherry-luc control (shC) or GSDMB-silenced (sh1) cells and treated with AbGB-NCs or NCs loaded with an irrelevant IgG (IgG-NCs). The scale bar represents the luciferase intensity (arbitrary units). **B–D**, Quantification of luciferase bioluminescence (**B**), tumor volume (**C**, images show the tumor size *ex vivo* of shC tumors after different treatments), and the final tumor weight (**D**) for the experiments shown in **A**. Data represent the mean values \pm SEM from shC cells, $n = 7$ mice per treatment condition; sh1, $n = 5$ per condition. **E**, Representative HER2, PCNA, and TUNEL staining pictures (scale bar, 25 μ m) for each experimental condition. **F**, Quantification of the proliferative (PCNA expression) and apoptotic (TUNEL staining) index of tumors from the experiments shown in **A** to **E**. **G**, Representative Western blot showing GSDMB expression in HCC1954 tumors treated with AbGB-NCs or IgG-NCs (left), quantification of GSDMB expression relative to GAPDH in the tumors analyzed ($n = 5$ per condition, right). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

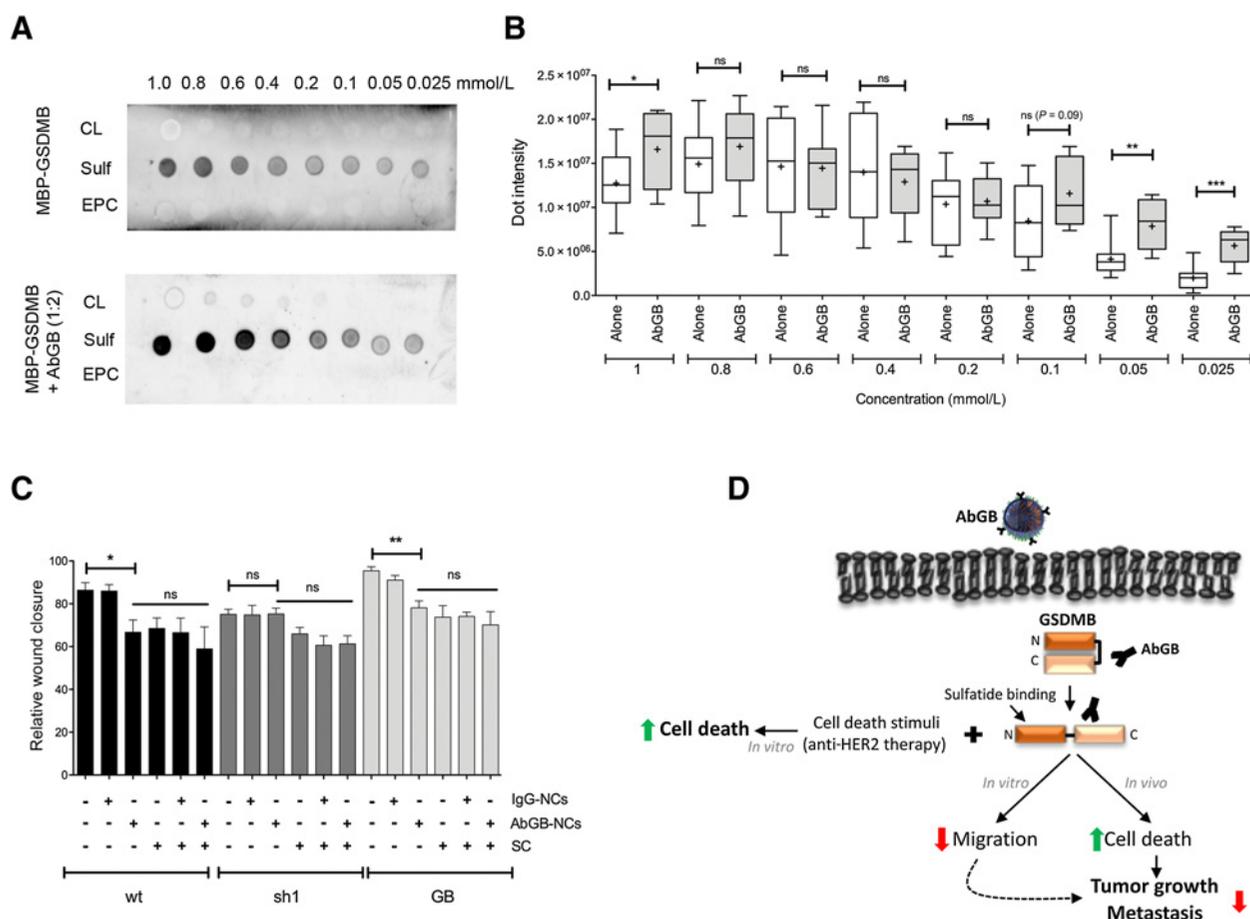
**Figure 5.**

AbGB-NC treatment reduces lung metastasis *in vivo* specifically in GSDMB-positive breast cancer cells. **A** and **B**, MDA-MB-231-HER2 cells with stable overexpression of GSDMB (GB) or the empty vector (C) were injected in the tail vein. After 1 week, mice were inoculated twice a week intraperitoneally with 200 mg/kg of NCs loaded with 4.17 mg/kg of AbGB or IgG. After 3 weeks, **(A)** the development of lung metastases was visualized using bioluminescence imaging and **(B)** quantified by measuring photon flux. Bars represent mean values \pm SEM from 6 animals per condition. **C**, Percentage of mice exhibiting lung metastases after histologic examination. **D**, Lung metastatic nodules (arrows) were detected in paraffin-embedded sections stained with hematoxylin and eosin (20x) or anti-HER2 antibody (40x).

presentation of its N-terminal region to enhancing sulfatide-binding.

Moreover, to investigate further whether the biological effects of AbGB depend on GSDMB binding to sulfatides, we performed wound-healing assays in the presence of sodium chlorate (SC), a chemical that inhibits sulfatide synthesis by blocking sulfation and subsequently reduces cell migration (35). HCC1954 cells

expressing different levels of GSDMB (WT endogenous levels, reduced by shRNA or overexpressed) were treated with AbGB-NCs or IgG-NCs in the presence/absence of SC. We first corroborated that AbGB specifically reduced the migration of GSDMB-high cells (Fig. 6C). Importantly, this effect is no longer observed in cells pretreated with SC when sulfatides synthesis is inhibited (Fig. 6C). Sulfatide blockage reduced migration in every experimental

**Figure 6.**

AbGB-NC treatment increases the binding of GSDMB to sulfatide. **A**, Protein lipid overlay assays. Purified recombinant MBP-GSDMB protein (6 $\mu\text{g}/\text{mL}$) was incubated in the presence or absence of anti-GSDMB (AbGB, 12 $\mu\text{g}/\text{mL}$) and tested for its binding capacity to varying amounts (mmol/L) of immobilized cardiolipin (CL), sulfatide (Sulf), or EPC (egg phosphatidylcholine, as negative control) on nitrocellulose membranes. **B**, Quantification of MBP-GSDMB binding to decreasing amounts of sulfatide by measuring relative dot intensity. Box plots represent data (median value depicted as a line, and mean value depicted as "+") from a total of 10 dots per lipid concentration (GSDMB alone or GSDMB + AbGB). **C**, The reduction in cell migration produced by AbGB-NCs is blocked in the absence of sulfatide. Wound-healing assays of HCC1954 wild-type (wt), GSDMB-silenced (sh1), or cells stably overexpressing GSDMB (GB). Cells were treated daily for 2 hours successively with SC (100 mmol/L) and then with 3.2 mg/mL of NCs loaded with anti-GSDMB (AbGB-NCs) or an irrelevant antibody (IgG-NCs). Quantification of the wound area covered by migrating cells at 72 hours with respect to 0 hour. The data represent the mean \pm SEM of three independent experiments. **D**, Representative scheme of the proposed mechanism of action of AbGB-NC treatment and the subsequent biological effects on GSDMB⁺ cancer cells. The GSDMB protein is represented in closed/autoinhibited and open conformations (N, N-terminal region; C, C-terminal domain). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant.

condition, and the combination of AbGB-NCs and SC had no significant additive effect. These results suggest that AbGB increases GSDMB-sulfatide binding and that this interaction is important for reducing GSDMB-mediated cell migration.

Overall, taken together all the above-mentioned results, we have provided a model (Fig. 6D) by which intracellular AbGB could mediate its biological effects, focusing in particular in GSDMB-sulfatide interaction.

Discussion

Identifying and validating new molecular targets, which are altered specifically in cancer cells, is essential to improve the survival of cancer patients. It is also crucial to tackle those molecular targets through modern technological approaches. In this work, we have addressed these issues by validating GSDMB

overexpression as a new molecular target in HER2 cancers and attacking this alteration with an innovative approach: the intracellular delivery of a functional antibody by nanotherapy.

Our previous data (8, 9), and the results shown in this study, prove that GSDMB overexpression/amplification promotes aggressive behavior in multiple ways (stimulating cell migration/invasion, metastatic behavior, drug resistance to anti-HER2 therapy, and poor prognosis) in HER2 breast cancers, independently of the hormone receptor status (8). Therefore, GSDMB cytoplasmic overexpression, which is indeed not observed in normal breast tissue (9), stands out as an attractive therapeutic target. Although reducing GSDMB expression by shRNAs has some effects on cancer cells *in vitro* (decreasing migration and partly sensitizing to trastuzumab; refs. 8, 9), here we proved that the protein targeting with a specific antibody (AbGB; ref. 8) delivers a full repertoire of therapeutic effects *in vitro* and *in vivo*

(Fig. 6D) including the blockage of tumor growth and metastatic potential *in vivo*. These therapeutic effects of AbGB-NCs require high levels of GSDMB, thus supporting that AbGB might modulate different biological mechanism than those mediated by shRNA reduction of total protein level. In this sense, one important function of Gasdermin proteins (procell death effect) depends on their structural conformation and, specifically, requires the release of their N-terminal autoinhibition (19, 33, 36, 37). After specific stimuli (such as viruses, bacterial infections, or chemotherapeutic agents), the gasdermin N-terminus domain binds to certain lipids in the plasma membrane and organelle (lysosomes, mitochondria), for instance phosphoinositides or cardiolipin (13, 14, 32). Using *in silico* bioinformatics and protein lipid overlays assays *in vitro*, our results suggest that AbGB-GSDMB binding alters its 3D protein configuration possibly resulting in the strengthening of the interaction of GSDMB (through its N-terminal region) with lipids, specifically sulfatides (33). Based on these results, we are proposing that AbGB biological effects may be mediated in part by an increased GSDMB-sulfatide binding, under certain circumstances. Sulfatides are involved in multiple biological functions in cancer (reviewed in ref. 34). In particular, sulfatide blockage decreases breast cancer cell migration and metastasis through different mechanisms, highlighting leptin binding (38) or integrin alpha V regulation (39). Based on motility assays data after sulfatide blockage, our working hypothesis (Fig. 6D) suggests that GSDMB binding to sulfatide modulates HER2 breast cancer-migratory behavior, possibly by an interaction/competition of GSDMB with promigration proteins or regulatory pathways.

Regarding the AbGB effect on increased trastuzumab sensitivity, we propose that under this cytotoxic stimulus, the increased GSDMB-sulfatide binding might release its pyroptotic effect (17) and subsequently amplify cell death signaling. Likewise, during tumor growth *in vivo*, the presence of stress stimuli (e.g., hypoxia, antitumor immune defense, stromal reaction, and growth constraints, among others) in combination with AbGB treatment would result in an enhanced cell death (as exemplified by the significant increase in TUNEL staining *in vivo* in tumors from two HER2/GSDMB⁺ cancer cell models). These ideas are quite consistent with the functional role of GSDME, which is activated upon chemotherapy and oncologic targeted therapies, and which triggers a cell death mechanism that is secondary to apoptosis induction (40). However, in contrast to GSDME activation, which requires N-term cleavage to bind lipids (40), we propose that increased lipid binding of the N-term domain from the full-length GSDMB would be enough to unveil its procell death function. In this case, GSDMB would work in the same way as mutant Gsdma3 does, which can promote cell death independently of cleavage (19, 41, 42). However, it is still unknown whether activated GSDMB may produce membrane pores and *bona fide* lytic cell death (pyroptosis) or induces other kind of intracellular damages (ROS, organelle dysfunction) in a similar way to other Gasdermins (13, 14, 32).

Aside these mechanistic considerations, our study is actually the first report which confirms that a Gasdermin protein can be used as a therapeutic target in cancer, and that our nanotherapy does effectively reduce many of the GSDMB protumor functions, including resistance to therapy. In this sense, we suggest that combining AbGB-NCs with trastuzumab could be a new treatment option not only for patients with aggressive drug-resistance breast cancers, but also for other tumors with HER2 overexpres-

sion, like gastric cancers (43), where GSDMB has been also detected (11, 44). Moreover, we hypothesize that the addition to other anti-HER2 antibodies (e.g., pertuzumab) or other chemotherapeutic agents to our anti-GSDMB nanotherapy could have a synergistic effect on cancer cell death. In fact, one of the possible developments of our nanotherapy could be the potential combination of our AbGB together with those anticancer agents into the same nanoparticles. Likewise, in this scenario, it would be desirable to test them into more clinically relevant *in vivo* models (45) such as HER2/GSDMB⁺ patient-derived xenografts.

In addition to the biological significance of this study, our work provides new methodological and technical advances in the field of nanotherapy. To the best of our knowledge, this is the first report showing *in vivo* therapeutic targeting of intracellular proteins with mAbs, without having altered their chemical structure or combining them with other anticancer drugs (46, 47). For this, we overcome those nanomedicine barriers, which do restrict the effective *in vivo* transfer of functional mAbs against cytosolic proteins, such as cell internalization inefficiency, poor stability in culture medium or plasma, nonspecific toxicity, and its delivery hardships into the target cancer cells *in vivo*. For instance, specific characteristics of our NCs, and in particular the functionalization with HA, produce stable, nonimmunogenic, biocompatible nanocarriers that are less toxic *in vitro* and *in vivo* than NEs. Moreover, although both NCs and NEs are internalized *in vitro* by endocytosis with similar effectiveness, NCs have a superior capability to target and accumulate in tumor tissue *in vivo*. Another key factor for the success of our AbGB-NC nanotherapy is that we have achieved a stable NC association with high amounts of antibody through physicochemical interactions, without requiring chemical reactions or other modifications. Furthermore, this association allows an efficient antibody internalization, which subsequently gets into the vesicle intracellular trafficking and is partly delivered into the cytosol (site of the target protein), possibly through an endosomal escape mechanism (48). Importantly, unlike irrelevant IgGs, the intracellular delivered AbGB remained functional for an extended time and incites multiple therapeutic effects, specifically on GSDMB-positive cells *in vitro* and *in vivo*.

In summary, our findings prove for the first time the workability of targeting GSDMB as a new therapeutic agent in HER2 cancer patients with poor clinical outcome. In addition, this novel approach will bring along the opening of new avenues in nanomedicine especially those designed to reach intracellular oncoproteins.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Á. Molina-Crespo, D. Sarrio, M. Gámez-Chiachio, A. Gonella, P.K. Dhal, M. Rodríguez-Serrano, M.L. García Bermejo, J.C. Triviño, A. Cano, G. Moreno-Bueno

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Study supervision: Á. Molina-Crespo, M. García-Fuentes, D. Torres, M.J. Alonso, G. Moreno-Bueno

Other (Generating and biochemically characterized the nanocapsules): A. Olivera

Other (Immunohistochemical analysis): E. Díaz

Other (Immunohistochemical analysis of samples): J. Palacios

Acknowledgments

This work was supported by grants from the Instituto de Salud Carlos III (ISCIII) and FEDER (PI16/00134), CIBERONC (CB16/12/00295), and the AECC (Grupos Estables de Investigación 2018-AECC) to G. Moreno-Bueno.

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Intracellular Delivery of an Antibody Targeting Gasdermin-B Reduces HER2 Breast Cancer Aggressiveness

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Clin Cancer Res 2019;25:4846-4858. Published OnlineFirst May 7, 2019.

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